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Development of in vitro Chylomicron Assay Using Caco-2 Cells

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Development of *in vitro* Chylomicron Assay Using Caco-2 Cells

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by

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ABSTRACT

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Dietary fats are mainly transported by the intestine in lipoproteins: chylomicrons (CMs) and very low density lipoproteins (VLDLs). Unfortunately, studies of the intestinal absorption of dietary fat have been hampered by the lack of an adequate *in vitro* model system. As an *in vitro* model Caco-2 cells are able to secrete lipoproteins. We investigated the possible factors that may affect the secretion of CMs through the ultracentrifugation technique. The dose-dependent effects of oleic acid, mono-olein, egg lecithin, collagen matrix, and the effect of cell differentiation on CM secretion were then tested. We found that oleic acid, lecithin, and cell differentiation are critical for CM secretion by Caco-2 cells. To further confirm that our optimal condition is, in fact, favorable for efficient CM production, we compared it with control groups. We observed that our condition led to more efficient CM secretion as determined by the TGs, ApoB, and TEM analysis.

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CHAPTER 1

INTRODUCTION

Obesity has been in a dramatic increase in the United States. About one-third of U.S. adults are obese and approximately 17% of children and adolescents are obese in 2010 according to Centers for Disease Control and Prevention (36). Unregulated food intake is known to be one of the main causes of obesity. Dietary lipids, the main composition in a western diet pattern, are absorbed by the small intestine and packed into 2 types of lipoproteins for transportation: CM and VLDL. However, the difference between CM and VLDL is not clearly understood including their different roles in developing obesity. In our study the production of CM is optimized *in vitro* to provide a tool for lipid-related studies.

Lipid Digestion

Before being absorbed by the small intestine, dietary lipids are processed in sequential steps: emulsification, hydrolysis, and micellization. Multiple processes are used to increase the surface area of the lipid droplets. First, physical force tears the lipid droplet down when stomach chime is propelled into duodenum through a small opening of pyloric orifice. Then emulsification further breaks the lipids droplet through amphipathic bile in the small intestine. The hydrophobic portion of amphipathic bile interferes with the lipids on the surface of lipid droplets and weakens the stability of lipid droplets which are broken down into smaller lipid droplets. Two enzymes, pancreatic lipase and phospholipase A2, hydrolyze dietary lipids and liberate hydrolysis products from lipid droplets. Further being mixed with bile, hydrolysis products are packed into micelle to pass through the unstirred water layer above the brush border membrane and taken up by enterocytes after micelles are disintegrated (3).

The importance of bile to emulsification and micellization is indicated by the decreased rate of lipid absorption in humans with bile fistulas (20). Such study indicates that bile in the duodenum is important for lipid absorption if not absolutely necessary. However, elevated concentrations of bile are shown to inhibit pancreatic lipase activity (14).

Digestion of Triglyceride

About 95% of dietary lipids are TGs while 5% consist of cholesterol, phospholipids, and other lipids. TG is digested by pancreatic lipase producing sn-2 monoglyceride (MG) and free fatty acids. sn-2 MG is the predominant form of TG hydrolysis products when other forms of MG could not be absorbed by enterocytes (32).

Digestion of Phospholipids

Dietary phospholipids are hydrolyzed by pancreatic phospholipase A2 into fatty acids and lysophospolipids in the intestinal lumen before absorbed by the enterocytes. Like TG, phospholipids hydrolysis products are esterified back into phospholipids and packed into lipoprotein as structural outer layer. Phospholipase A2 knockout mice are indistinguishable from wild-type controls when fed regular chow except for their resistance to diet-induced obesity (3).

Uptake of Monoglyceride and Free Fatty Acid

While MG is taken up through protein-independent diffusion, uptake of fatty acids is likely through both protein-independent diffusion and protein-dependent transportation. The evidence for passive diffusion is that the uptake of fatty acid is not affected by protease treatment *in vitro* (23). Similar result were reported in adipocytes when flip-flop mechanism was found to transport fatty acids across the apical membrane of epithelial cells (22). The evidence for active

transport is that fatty acid uptake can be saturated and there are competitions for uptake between different fatty acids. A number of candidate proteins are proposed to function in protein-dependent transportation. First, FAT/CD36, highly expressed in enterocytes, was reported to have effects in the uptake of fatty acids (7). The expression of FAT/CD36 is upregulated by dietary fat. However, the controversy also exists because only the secretion of lipoproteins is impaired in CD36-null animals but the uptake of fatty acid is not (9). The second candidate is FATP. FATP has 6 different proteins in which FATP4 is predominantly expressed in the intestine. FATP is believed to help facilitate the uptake of fatty acid by the intestine, supported by the fact that 40-50% of fatty acid uptake is reduced in knockdown animals (27). The third candidate is FABPpm that has been localized on the brush border membranes of enterocytes. The antibodies to FABPpm also reduce the fatty acid uptake (15).

Assembly of Triglyceride in Enterocytes

Through either diffusion or protein-dependent transport, TG hydrolysis products are absorbed by enterocytes and traverse into endoplasmic reticulum (ER) for reesterifying back into TG. Two binding proteins to traverse TG hydrolysis products in enterocytes are liver FABP and intestinal FABP(4). Inside the ER, TG is assembled in 2 pathways: MG pathway and glycerol-3-phosphate pathway. The MG pathway covalently joins MG and fatty acyl-CoA to form diglyceride (DG) by MGAT (5) and further acylation of DG by DGAT leads to the production of TG. The MG pathway is the major pathway for TG biosynthesis, especially predominant when sufficient lumen MG and fatty acids are supplied in the postprandial state. Two DGATs are identified and characterized: DGAT1 and DGAT2. Lipid absorption is impaired in DGAT2-knockout animal but not DGAT1-knockout animal (35). Another pathway, glycerol-3-phosphate pathway, is used to assemble TG when lumen MG and fatty acid are not available in the fasting

state. The addition of 2 fatty acyl CoAs to glycerolphosphate leads the production of phosphatidic acid catalyzed by glycerol-phosphate acyltransferase (GPAT) and acyl-glycerol-phosphate acyltransferase (AGPAT). Phosphatidate is then dephosphorylated by phosphatidic acid phohydrolase (PAP) to yield DG, and another fatty acyl CoA addition leads to TG (37) (See Figure 1).

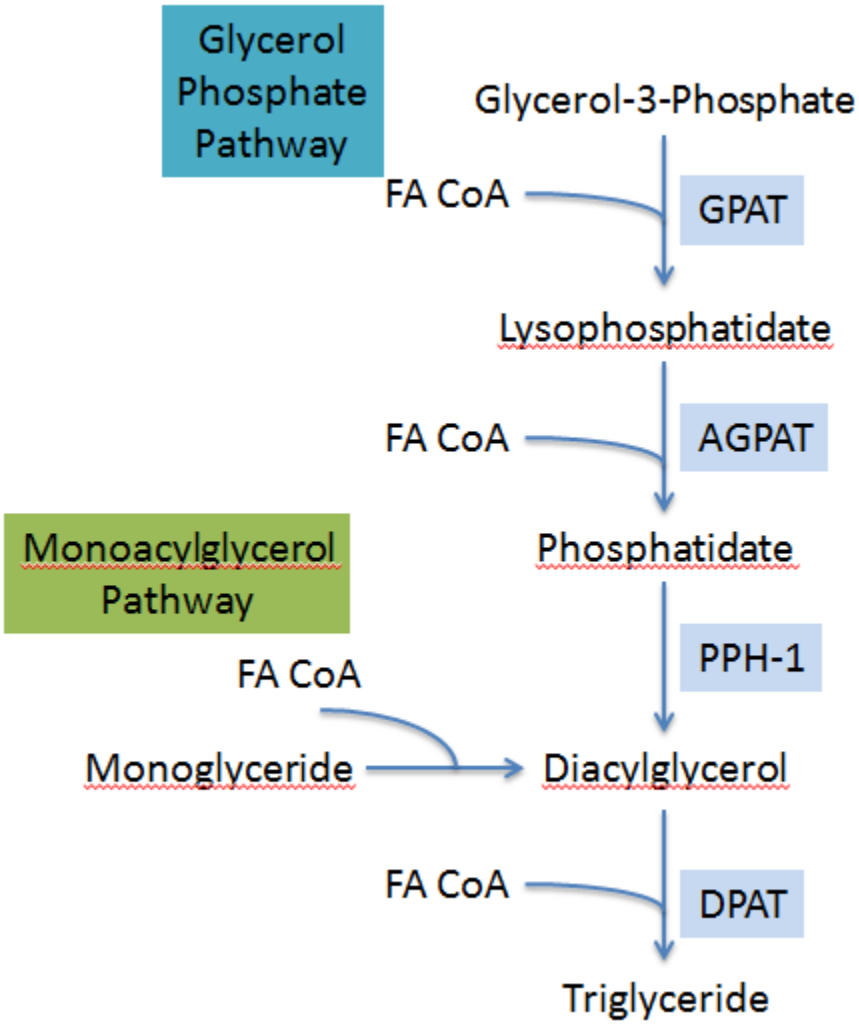


Figure 1. Two Pathways for TG Synthesis: MG pathway and glycerol-3-phosphate pathway. In MG pathway, MG is acylated by MGAT and DGAT to assemble TG. In glycerol-3-phosphate pathway, glycerol-3-phosphate is joined by 2 FA CoA catalyzed by GPAT and AGPA and dephosphorylated by PAP to yield DG. Addition of FA CoA to DG yields TG (adapted from Yen 2008)

Lipoprotein Production

After being absorbed by enterocytes, lipids hydrolysis products are reesterified and packaged into lipoproteins to be transported in hydrophilic environment. There are some key proteins that are known to affect intestinal lipoprotein secretions: Apolipoprotein B-48, microsomal TG transfer protein (MTP), and ApoA-IV.

Apolipoprotein B-48

ApoB is essential for lipoprotein assembly in the small intestine and there is only one ApoB per lipoprotein (1). Two forms of ApoB, intestine-only ApoB-48 and ApoB-100, are encoded by the same gene. In order to produce ApoB-48 in the intestines, nucleotide of ApoB mRNA is converted from a cytidine to urideine. As a consequence, a stop codon replaces original glutamine, leading to only the NH_2-terminal mRNA translated. Therefore, ApoB-48 lost the ability to bind LDL-receptor with only 48% of apoB100 left. Besides this, lipoprotein with ApoB-48 is also proved to be a better transporter under high concentrations of lipids (23). Fatty acid could dramatically increase the secretion of ApoB-48 contained lipoproteins without mRNA of ApoB increased (17). It is believed that ApoB-48 synthesis is consecutive and constant. In preprandial state, ApoB-48 without being lipidated would be degraded by the ubiquitin-proteasome system after assembled due to lack of fatty acids and in the postprandial state increased fatty acids with similar ApoB amounts lead to an increase in size of lipoproteins.

Microsomal Triglycerides Protein

MTP is used for transportation of newly-synthesized TG to newly translated ApoB in the ER lumen of small intestine and liver. The initial lipidation of ApoB by MTP protects ApoB from proteasome degradation. The large subunit of MTP is responsible for lipid transfer subunit

and the small subunit of MTP is identical to protein disulfide isomerase (PDI) (18). Abetalipoproteinemia is caused by mutation in the large subunit of MTP that is characterized by an inability to secrete intestinal chylomicrons or hepatic VLDL.

Apolipoprotein A-IV

Apolipoprotein A-IV (Apo A-IV), expressed predominantly in the small intestine, is a lipid binding protein. Despite numerous functions that have been described, ApoA-IV has a primary role in the intestinal lipid absorption. As the most responsive to intestinal lipid flux, Apo A-IV is incorporated into nascent chylomicrons in the ER and is secreted on the surface of chylomicrons (30).

Chylomicron vs. Very Low Density Lipoprotein

CM and VLDL are lipoproteins secreted by enterocytes when CM has a diameter of 80 nm or larger and VLDL is smaller than 80 nm in diameter. VLDL is predominant in the fasting state and more CM is secreted in the postprandial state. CM is considered as a more efficient lipid transporter than VLDL. In the preprandial state, only 0.7% of lymph lipoproteins were CMs. In the postprandial state, around 39% of lymph lipoproteins were CMs for dietary fat transportation (26). Pluronic 81 is capable of blocking the production of CM from Caco-2 cell but not the production of VLDL. This suggests that the production of CM and VLDL use different pathways (10).

Chylomicron Production

Formation of chylomicron has multiple steps. The first step, ApoB-48, synthesized in rough endoplasmic reticulum (RER), is chaperoned by MTP. Newly synthesized ApoB-48 is

either assembled with cholesterol, phospholipids, and small amounts of TG to form stable complex dense particles (DP) or is degraded when lipids are not available. Then in smooth endoplasmic reticulum (SER), MTP carries TG and cholesterol ester to enlarge the particle that merges with Apo A-IV (18). Then prechylomicron is formed that contains a core of neutral lipids surrounded by a monolayer of phospholipid with ApoB-48 and ApoA-IV. L-FABP may facilitate budding of the preCM transport vesicle (PCTV). The PCTV fuses to the *cis*-Golgi via coating protein II and soluble *N*-ethymaleimide-sensitive factor attachment protein receptor (SNARE) protein. After final processing in the Golgi, the mature CM is secreted via the basolateral membrane (2).

Physiology of the Intestinal Mucosa

Epithelium cells of the small intestine secreted lipoproteins from basolateral membrane into lamina propria where there are both lymphatic capillaries (lacteal) and blood capillaries (fenestrated capillaries). The large gaps between lacteal are considered much larger than the size of lipoproteins so that all lipoprotein are assumed to enter the lymphatic system. That is the reason lipoproteins studied *in vivo* are collected from the lymph. However, the pore size of the fenestrated capillaries is about 60-80 nm in diameter. In addition to fast flow in blood capillaries, some VLDLs enter the blood capillaries and are taken up by the liver.

Caco-2 Cells

Caco-2 cells derived from a human colorectal carcinoma could differentiate into enterocyte-like function and morphology such as apical microvilli, associated brush border hydrolase, sucrose-isomaltase that is an enterocyte-specific differentiation marker and functional tight junction barrier that is indicated by high transepithelial electrical resistance (17). Sucrase

ctivity can be detected after confluence and gradually increase until 14-20 days postconfluent. Some chemicals like collagen matrix are known to affect the growth of Caco-2 cells (2).

Apolipoprotein B 48 vs. Apolipoprotein B-100 from Caco-2 Cells

Despite that Caco-2 cells are intestine-derived, it can assemble both intestinal and hepatic forms of ApoB. Some studies showed that more hepatic-only ApoB-100 is secreted than ApoB-48 (24). However, the ratio of ApoB-48/ApoB-100 can be affected by growth and differentiation. Caco-2 cells predominantly produced ApoB-100 when cultured directly on plastic plates (24). But when cultured on filter or semipermeable membranes, Caco-2 cells synthesize more ApoB-48 than ApoB-100 (13). The difference is probably due to the degree of polarization or maturation based on different growth supports. Besides Caco-2 cell line model, small intestinal explants model also produces ApoB-100, implying that mRNA editing to ApoB-48 is incomplete (21).

Criticism on Caco-2 Cells

There are also criticisms for using Caco-2 cells as an *in vitro* model. The primary criticism is their poor lipid secretion efficiency, particularly their CM secretion efficiency. Most studies associated with CMs had to use sensitive test methods, e.g. radiolabeled lipids. In our studies, we optimized the conditions for Caco-2 cells to secrete CMs so that the lipids secreted can be detected by colorimetric assay and the particles can be visualized by TEM.

CHAPTER 2

MATERIALS AND METHODS

Caco-2 Cells

The human adenocarcinoma, Caco-2, cells were obtained from American Type culture Collection (Rockville, MD), and grown and maintained with growth media (DMEM with 15% FBS) in 5% CO_2 at 37°C. Cells were split at 1:6 ratio when they reached 50-70% confluent, and media was changed every other day. To determine optimal condition for CM secretion, cells were maintained in 10 cm culture dishes until they reached at least 13 days postconfluence. To further comfirm the optimal conditional, cells were cultured on Tranwellsand its condition was compared with the commonly reported condition (25). The prefiltered lipid mixture (unless specified, Oleic Acid: Lecithin: NaTC = 2 mM: 1.36 mM: 1.0 mM) in 10 ml growth media was added to the cells to induce the secretion of CMs. Cells that were grown on culture dishes were incubated with lipid mixture for 4 hours; washed with PBS twice, and replaced with fresh growth media to collect the secreted lipoproteins. Cells grown in Transwells were incubated with lipid mixture in apical chamber for 4 hours: the basolateral chamber was added with fresh growth media (no lipid mixture) to collect the secreted lipoproteins.

Sequential NaCl Density Gradient Ultracentrifugation

Sequential NaCl density gradient ultracentrifugation was used to isolate CMs and VLDLs from the basolateral media of the Caco-2 cells. Media collected from cells were mixed with NaCl and water to obtain the density of 1.20 g/ml. The 1.20 g/ml density mixture was then carefully overlaid with 500 µl of water and subjected to sequential ultracentrifugation. The top 500 µl (CM fraction) was isolated after 30 minute spin at 10,000 rpm (Thermo T865 rotor). The

remaining mixture was similarly overlaid with 500 µl of water and spun at 65,000 rpm for 24 hours. The top 500 µl of VLDL fraction was isolated by gentle pipetting.

Fat Red 7B

For the purpose of visualizing the lipid layers on the ultracentrifugation samples, colorless media (without the pH indicator phenol red) was used to collect the secreted lipoproteins. The colorless lipoprotein-containing media was added with 200 µl of Fat Red 7B solution (2 mg/ml Fat Red 7B in 0.1M NaOH with 1 drop of Triton) and were subjected to sequential NaCl gradient ultracentrifugation as described above. Digital pictures were taken after each spin. With the exception of this experiment, no other experiments used colorless media for Fat Red 7B staining.

Enzyme-Linked Immunosorbent Assay (ELISA)

ApoB was quantified by a sandwich ELISA method. High binding 96-well plates (Thermo Scientific Cat# 62409-002) were coated with monoclonal anti-ApoB antibody (Thermo Scientific Cat#HYB069-02-02; 100 µl in each well; 1:5,000 dilution in Voller's buffer (2.76g Na_2CO_3, 1.916g $NaHCO_3$, 0.2g NaN_3 in 1L, pH=9.2)) followed by blocking with 5% BSA in PBS-0.5% Tween (PBS-T). After coating, each well was washed 3 times with PBS-T. 100 µl of ApoB standard (Alpha Diagnose Cat# APOB25-N-100) or samples were added into each well and incubated. Each well was then washed 3 times and incubated with the goat anti-ApoB antibody (diluted in 1:2000; Rockland Cat# 600101111). After being washed 5 times, each well was incubated with anti-goat horseradish peroxidase-conjugated secondary antibody (diluted 1:2,000; Thermo Scientific Cat# 31402). Peroxidase substrate (Bio-Rad Cat# 172-1064) was added and the absorbance was read according to manufacturer's suggested protocol.

Triglycerides Measurement

The concentration of TGs was measured by using colorimetric assay, as described previously (33).

Transmission Electron Microscopy

Samples were negatively stained by using freshly prepared 2% phosphotungstic acid, pH 6.0, as previously described (33). They were then examined by using Philips Technai 10 and representative pictures were taken.

Optimizing Chylomicron Secretion

Variables including differentiation stage, lipid mixture composition, lipid mixture incubation time, lipoprotein collection time, and collagen matrix were tested.

Differentiation Stage

Caco-2 cells that have reached 4, 13, 17, and 23 days postconfluent were used to study the effect of differentiation stage on lipoprotein secretion. They were incubated with prefiltered lipid mixture (oleic acid: egg-lecithin: NaTC= 4 mM: 1 mM: 0.68 mM: 1.0 mM) in growth medium for 4 hours. The lipid mixture was then removed; the cells were washed twice with PBS, and incubated with fresh growth media for 2 hours. The collected media were subjected to sequential NaCl gradient ultracentrifugation for the isolation of the lipoprotein fractions followed by TG analysis, as described above.

Lipid Mixture Composition

The dose-dependent effects of the hydrolysis products of dietary lipids on lipoprotein secretion were studied. The hydrolysis products that we tested included oleic acids, mono-olein, and egg-lecithin. Caco-2 cells were used when they were between 13 days and 15 days postconfluent. Cells were incubated with lipid mixture (oleic acid: mono-olein: egg-lecithin: NaTC= 2 mM: 1.32 mM: 1.0 mM) in 10 ml of growth media for 4 hours, washed twice, followed by incubation with fresh growth media for 2 hours. Similarly, the collected media were subjected to sequential NaCl gradient ultracentrifugation for the isolation of the lipoprotein fractions followed by TG analysis.

For the oleic acid experiment, 0, 2, 4, and 6 mM of oleic acids in 1mM mono-olein, 0.68mM lecithin, and 1mM NaTC in growth media were used. For mono-olein experiment, 0, 1, 2, or 4 mM of mono-olein in 2 mM oleic acids, 0.68 mM lecithin, and 1.0 mM NaTC in growth media were used. For the lecithin experiment, 0, 0.68, 1.36, or 2.72 mM of lecithin in 2 mM oleic acids and 1.0 mM NaTC in growth media were used.

Thin Layer Chromatography Analysis

The egg-lecithin was analyzed by TLC (33) to confirm the presence of lysophosphatidylcholine (lysoPC). Half milligram of lysoPC and 2 mg of egg-lecithin in chloroform were separated on silica gel 60 plates using chloroform : methanol : acetic acid : water (50/37.5/3.5/2) (v/v) as the solvent system. The plate was stained with choline staining (2 g potassium iodide, 4 ml acetic acid, 0.34 g bismuth subnitrate in 100 ml total volume) followed by 20% sulfuric acid staining with 225°F heating until the color developed.

Incubation Time and Collection Time

After Caco-2 cells have reached 13 days postconfluent, lipid mixture (2 mM oleic acids, 1.36 mM lecithin and 1.0 mM NaTC) in 10 ml growth media were added to the cells for either 2 or 4 hours. Cells were washed twice with PBS, and incubated with growth media for either 2 or 4 hours. The collected media were subjected to sequential NaCl gradient ultracentrifugation for the isolation of the lipoprotein fractions followed by TG analysis.

Collagen Matrix

Tissue culture dishes (10 cm) were precoated with 0, 50, 100, or 500µg collagen per plate according to manufacturer's suggested protocol (MP Biomedical Cat# 160084). After the cells have reached 13 days postconfluent, lipid mixture (2 mM Oleic Acid, 1.36 mM lecithin, and 1.0 mM NaTC) in 10 ml growth media were added to the cells and incubated for 4 hours. The cell were washed twice and incubated with fresh growth media for 2 hours. The collected media were subjected to sequential NaCl gradient ultracentrifugation for the isolation of the lipoprotein fractions followed by TG analysis.

Comparing the Optimal Condition with the Control Groups

Cells were cultured Transwell, as described previously (34). For the optimal condition (O), the preferred lipid mixture (2 mM oleic acids, 1.36 mM lecithin and 1.0 mM NaTC) in 10 ml growth media were added to the apical chamber and growth media without lipid were added to the basolateral chamber, followed by 4 hours incubation. For the condition with low lipid (LL), lipid mixture containing 1.6 mM oleic acid and 0.5 mM NaTC in 10 ml growth media were added to the apical chamber and growth media without lipid were added to the basolateral chamber, followed by 14 hours incubation. For the condition with no lipid (NL), growth media

without lipid were added to both the apical and basolateral chambers and incubated for 4 hours. The collected basolateral media were mixed with protease inhibitor and subjected to sequential NaCl gradient ultracentrifugation for the isolation of the lipoprotein fractions followed by TG, ELISA, TEM, and Western blot analysis. One-step NaCl density gradient ultracentrifugation was used instead. In one-step NaCl gradient ultracentrifugation, CMs and VLDLs were isolated as one single fraction by skipping the 30 minutes spin at 10,000 rpm.

Lipoprotein Particle Size Analysis

Lipoprotein samples from O, LL, and NL that were isolated by one-step NaCl density gradient ultracentrifugation were negatively stained with 2% phosphotungstic acid (pH= 6.0), as previously described (33). The size of the lipoprotein particles was measured through representative TEM pictures. For each group, at least 800 particles were counted per sample.

SDS Polyacrylamide Gel Electrophoresis and Immunoblotting

The lipoprotein fraction (10 µl) isolated by one-step NaCl density gradient ultracentrifugation method was run on the 4-20% polyacrylamide gel (Bio-Rad Cat# 456-1096) using the suggested manufacturer's protocol. The proteins were then transferred to nitrocellulose membrane and blocked with 5% skim milk in TBS-T for 30 minutes. The membrane was incubated with monoclonal antiApoB antibodies (Thermo Scientific Cat# HYB069-02-02; 1:5,000 dilution in blocking buffer). After 2 washes with TBS-T, the membrane was incubated with goat antimouse antibodies (Thermo Scientific Cat#LK152970; 1:2000 dilution in blocking buffer). After 5 washes, the signals were detected by using the HRP substrate kit (Thermo Scientific Cat# NG173486).

High-Performance Liquid Chromatography (HPLC)

100 µl of the lipoprotein fraction isolated by 1-step NaCl density gradient ultracentrifugation injected into the 100 µl sample loop of Bio-Rad Duoflow system and separated by gel filtration column (Shodex Cat# SB-804HQ). About 180 of 1 ml fractions were collected for analysis (buffer: 0.05 M NaCl).

Statistical Analysis

The data shown are mean values ± standard errors (SE). To determine the statistical significance of 3 groups or more, 1-way ANOVA was used. *t*-test was used for comparison between 2 groups. Statistical analyses were performed in Excel (Microsoft, Seattle, WA), and were considered significant if $P < 0.05$. $n>2$ for all experiments.

CHAPTER 3

RESULTS

Fat Red 7B Staining

As shown in Figure 2, the staining spread evenly in the unfractionated lipoprotein layer before the ultracentrifugation. After the first spin (Figure 2A) the stain was concentrated in the top ~ 1 ml layer, though the bottom layer was lightly stained as well. After the second spin (Figure 2B) the stain appeared strongly in the top ~ 1 ml layer and the bottom layer with the middle layer minimally stained.

A) B)

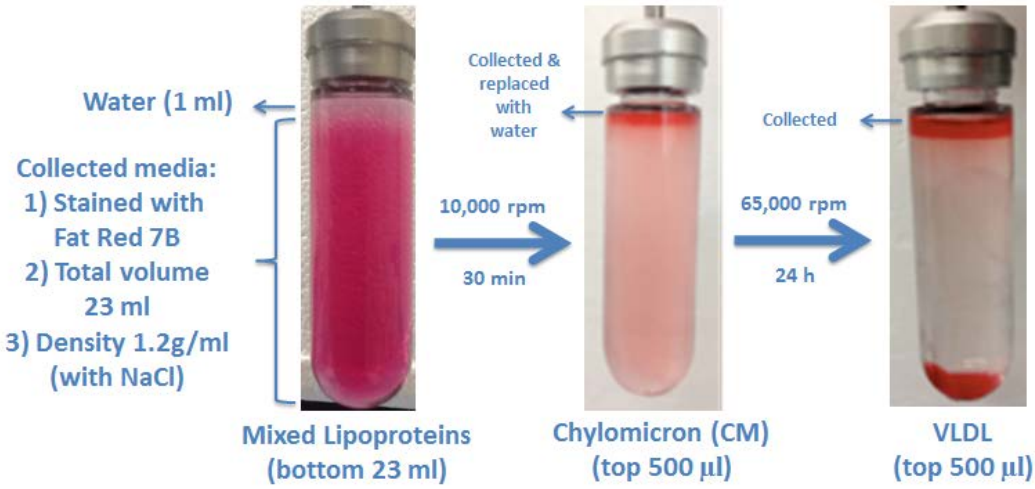


Figure 2. Fat Red 7B Staining

The heavily stained CMs (A) floated to the top ~ 1ml layer. Subsequently, the top 1 ml of the chylomicron fraction was replaced with 1 ml of water and spun for 24 hours at 300,000 g. The heavily stained VLDLs (B) were visible in the top ~ 1 ml layer.

Analysis on Sequential NaCl Density Gradient Ultracentrifugation

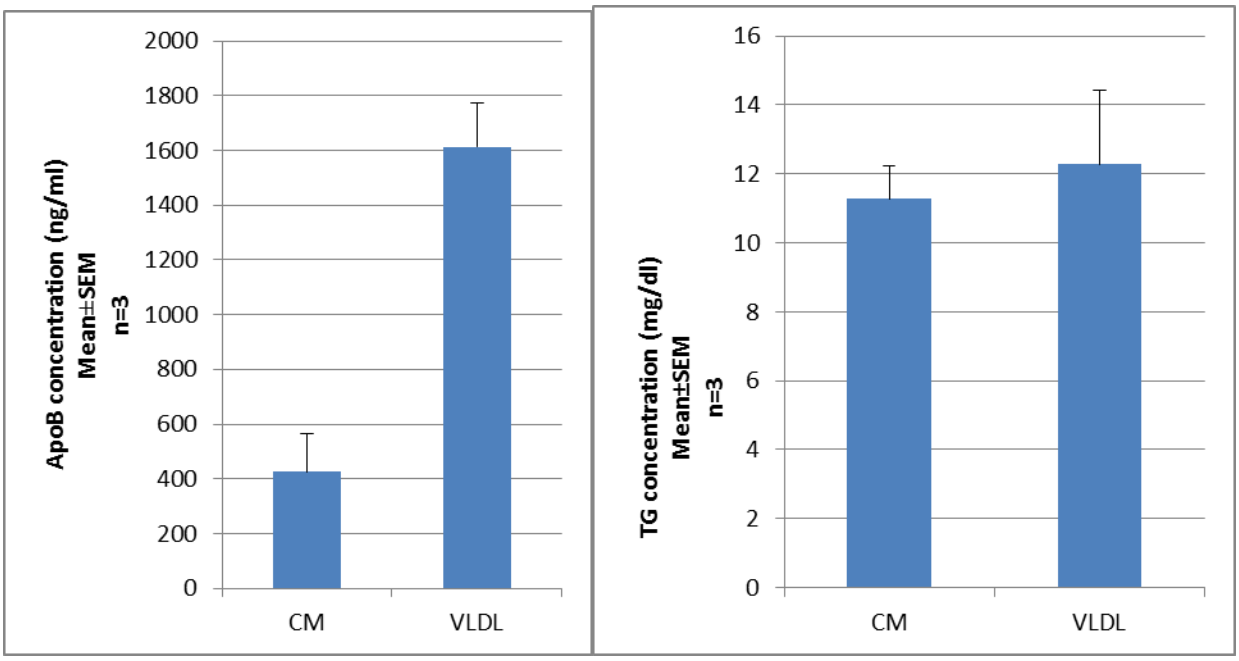
Data in Table 1 showed the difference between CM and VLDL on TG and ApoB. The concentrations of ApoB from the top layer of the first and second spins were, 425.3 ng/ml and 1612.18 ng/ml, respectively (p= 0.005). The concentration of TGs from the top layer of the first and second spins were 11.27 mg/dl and 12.27 mg/dl, respectively (p= 0.69).

Lipoprotein Fractions Isolated by Sequential NaCl Gradient Ultracentrifugation		
	ApoB (ng/ml)	TG (mg/dl)
CM	425.3 ± 139.2	11.27 ± 0.945
VLDL	1612.18 ± 160.56	12.27 ± 2.15

Table 1. Lipoprotein Fractions Isolated by Sequential NaCl Gradient Ultracentrifugation

As shown in Figure 3, both CM and VLDL fractions were analyzed for their Apolipoprotein B (A) (p= 0.005), TG (B) (p=0.69) and particle size using the transmission electron microcopy with negative staining method. Notice that the particles in the CM fraction (C) were larger than those in the VLDL fraction (D).

A) B)



C)

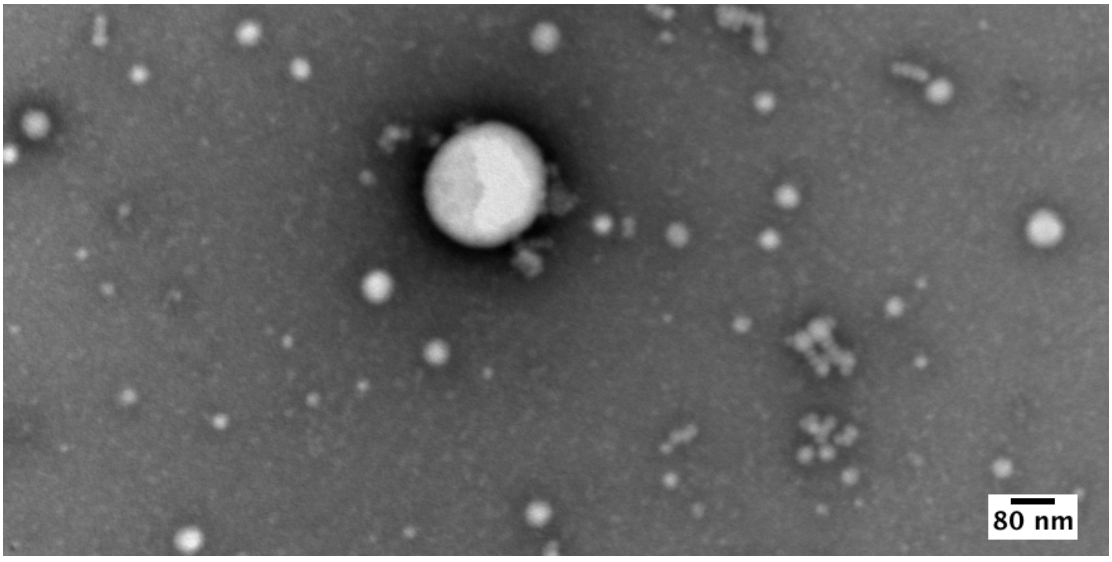


Figure 3 (continued on the next page)

D)

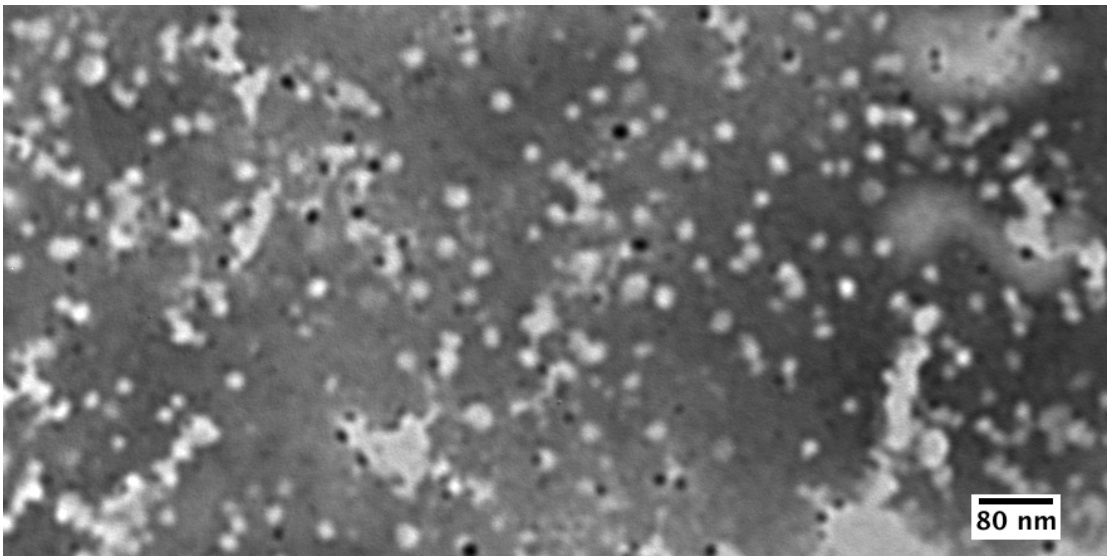


Figure 3. Lipoprotein Fractions Isolated by Sequential Gradient Ultracentrifugation

Optimizing Chylomicron Secretion: Differentiation Stage

Data in Table 2 show the effect of cell differentiation on CM and VLDL secretion.

The Effect of Cell Differentiation on Lipoprotein Secretion		
Days postconfluent (days)	CM (mg/dl)	VLDL (mg/dl)
4	5.99 ± 0.305	9.29 ± 1.39
13	8.19 ± 1.05	13.87 ± 2.46
17	11.81 ± 1.86	11.95 ± 1.80
23	7.09 ± 1.16	11.32 ± 1.87

Table 2. The Effect of Cell Differentiation on Lipoprotein Secretion

To determine the efficiency of lipoprotein secretion, TG concentrations from both the CM and VLDL layers were measured. As shown in Figure 4A, Caco-2 cells that have reached 13 and 17 days postconfluent were more efficient in CM secretion than those of 4 and 23 days postconfluent ($p= 0.0094$) but there was no significant difference between the 13 and 17 days postconfluent. Figure 4B shows that the efficiency of VLDL secretion was not significantly affected by cellular differentiation stage ($p=0.449$).

A) B)

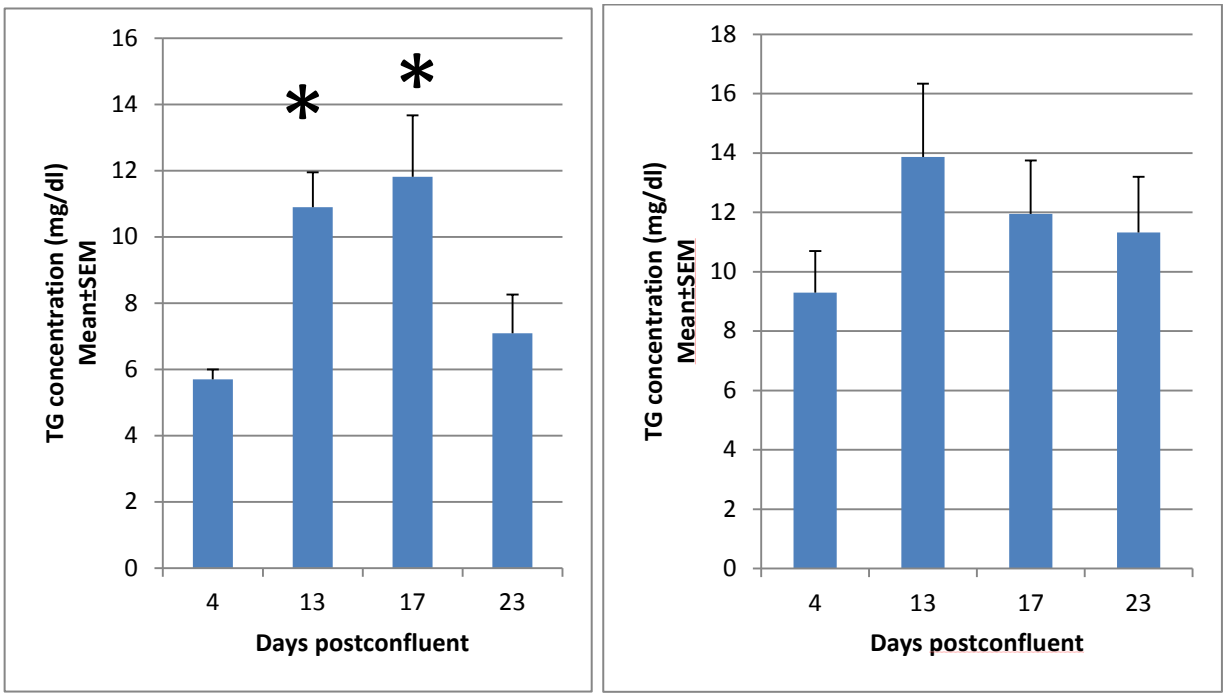


Figure 4. The Effect of Cell Differentiation on Lipoprotein Secretion

Caco-2 cells were grown on 10 cm plate until they reached 4, 13, 17, or 23 days postconfluent. The cells were then incubated with 4mM oleic acid, 1mM mono-olein, 0.68mM egg lecithin and 1mM NaTC in 10 ml growth media. After 4 hours of incubation, the cells were washed twice with PBS and replaced with fresh growth media. After 2 hours, the media was

collected. The collected media was subjected to sequential gradient ultracentrifugation. The triglyceride concentrations in the CM (A) (p= 0.0094) and VLDL (B) fractions were measured using a colorimetric assay. n =>3.

Optimizing Chylomicron Secretion: Oleic Acids

As shown in Table 3, oleic acids significantly affected the efficiency of chylomicron secretion (p= 0.0017) but not VLDL secretion (p= 0.679). 2 mM oleic acids resulted in the most efficient CM secretion. Our preliminary data showed that 0.5 and 1 mM oleic acids resulted in lower efficiency of chylomicron secretion than 2 mM oleic acids (data not shown).

Dose-dependent Effects of Oleic Acids on Lipoprotein Secretion		
OA Concentration (mM)	CM (mg/dl)	VLDL (mg/dl)
0	6.24 ± 0.37	8.992 ± 1.01
2	12.74 ± 0.82	12.04 ±1.23
4	10.08 ± 0.89	12.81 ± 1.44
6	8.08 ± 0.61	12.63 ± 2.79

Table 3: Dose-dependent Effects of Oleic Acids on Lipoprotein Secretion

These data showed the cell differentiation affected the CM secretion but not VLDL fraction. (See Figure 5)

A) B)

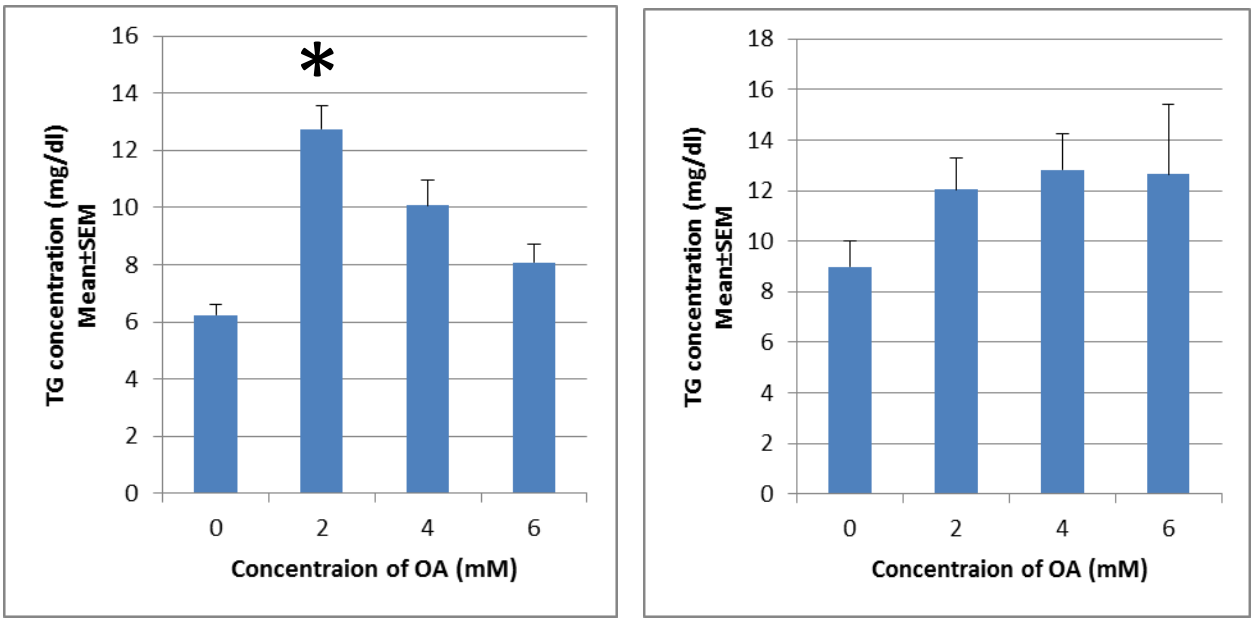


Figure 5. The Effect of Cell Differentiation on Lipoprotein Secretion

Caco-2 cells were grown on 10 cm plastic plate until they reached 13 day postconfluent. The cells were then incubated with varying amount of lipid. Cells were incubated with 0, 2, 4, or 6 mM of OA in 0.68 mM lecithin, 1 mM MG, and 1 mM NaTC. After 4 hours of incubation, the cells were washed twice with PBS and replaced with fresh growth media. After 2 hours, the media was collected. The collected media was subjected to sequential NaCl gradient ultracentrifugation. The triglyceride concentrations in the CM (A) ($p= 0.0017$) and VLDL (B) ($p=0.679$) fractions were measured using a colorimetric assay. n=>3

Optimizing Chylomicron Secretion: Mono-olein

As shown in Table 4, different concentrations of MG did not significantly affect the production of CM ($p=0.35$) or VLDL ($p=0.74$).

Dose-dependent Effects of Mono-olein on Lipoprotein Secretion		
MG concentration (mM)	CM (mg/dl)	VLDL (mg/dl)
0	8.906 ± 0.73	9.7 ± 0.87
1	10.09 ± 1.76	9.76 ± 0.90
2	7.79 ± 0.68	8.96 ± 1.07
4	5.75 ± 0.72	7.72 ± 1.30

Table 4: Dose-dependent Effects of Mono-olein on Lipoprotein Secretion

As shown in Figure 6, mono-olein did not significantly affect the efficiency of either chylomicron secretion (p= 0.358) or VLDL secretion (p=0.749), as shown in Figure 5A and 5B, respectively.

A) B)

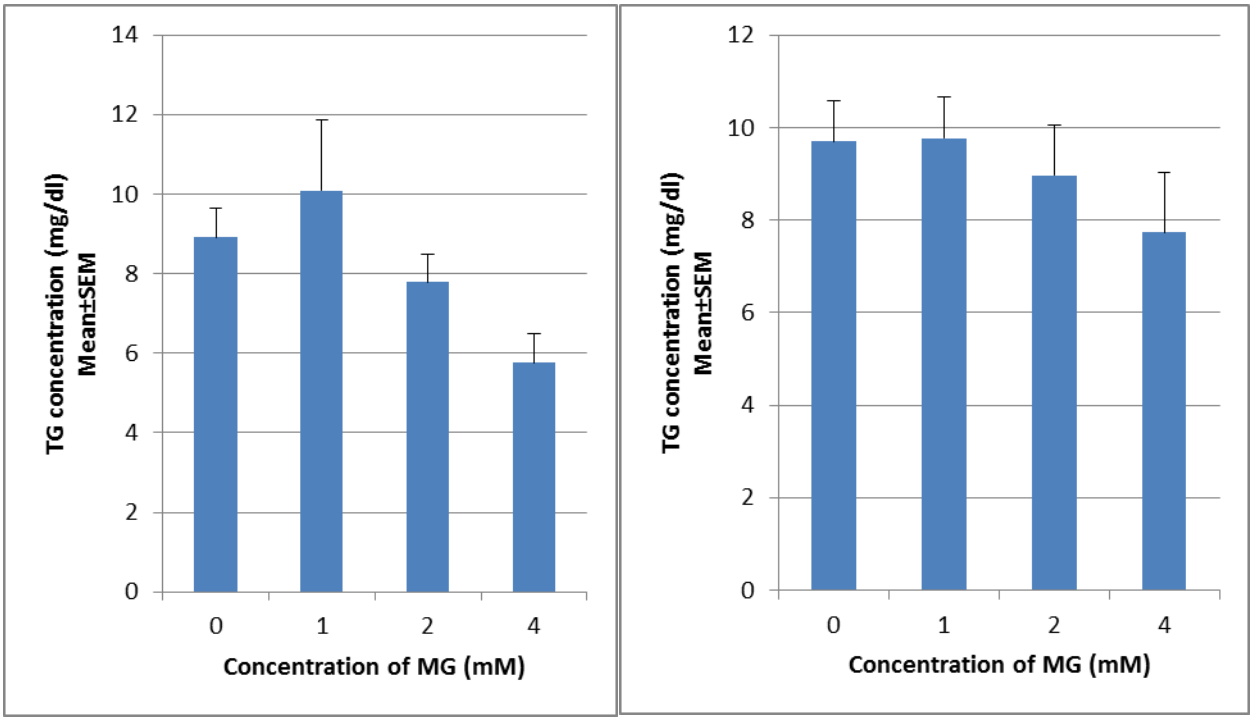


Figure 6. Dose-dependent Effects of Mono-olein on Lipoprotein Secretion

Caco-2 cells were grown on 10 cm plate until they reached 13 day postconfluent. The cells were then incubated with varying amount of lipid. Cells were treated with 0, 1, 2, or 4 mM MG in 2 mM oleic acid, 0.68 mM egg lecithin and 1mM NaTC. After 4 hours of incubation, the cells were washed twice with PBS and replaced with fresh growth media. After 2 hours, the media was collected. The collected media was subjected to sequential NaCl gradient ultracentrifugation and the TG concentrations in their CM (A) ($p= 0.358$) and VLDL (B) ($p= 0.749$) fractions were measured.

Optimizing Chylomicron Secretion: Egg Lecithin

As shown in Table 5, egg lecithin did significantly affect the CM production ($p=0.022$) but not the VLDL production ($p=0.053$) from Caco-2 cells. Concentrated egg lecithin induced Caco-2 cells to secrete significantly more CM.

Dose-dependent Effects of Egg Lecithin on Lipoprotein Secretion		
Lecithin Concentration (mM)	CM (mg/dl)	VLDL (mg/dl)
0	5.42 ± 0.49	7.2 ± 1.17
0.68	5.77 ± 0.53	9.59 ± 1.61
1.36	11.41 ± 1.69	13.52 ± 2.42
2.72	12.48 ± 1.99	15.68 ± 1.91

Table 5: Dose-dependent Effects of Egg Lecithin on Lipoprotein Secretion

Figure 7A shows that 1.36 mM and 2.72 mM lecithin led to more efficient CM secretion ($p=0.022$). However, there was no significant difference between the 1.36 mM and the 2.72 mM lecithin groups. As shown in Figure 7B, lecithin did not significantly increase the efficiency of VLDL secretion, though the trend was clearly present ($p= 0.053$).

A) B)

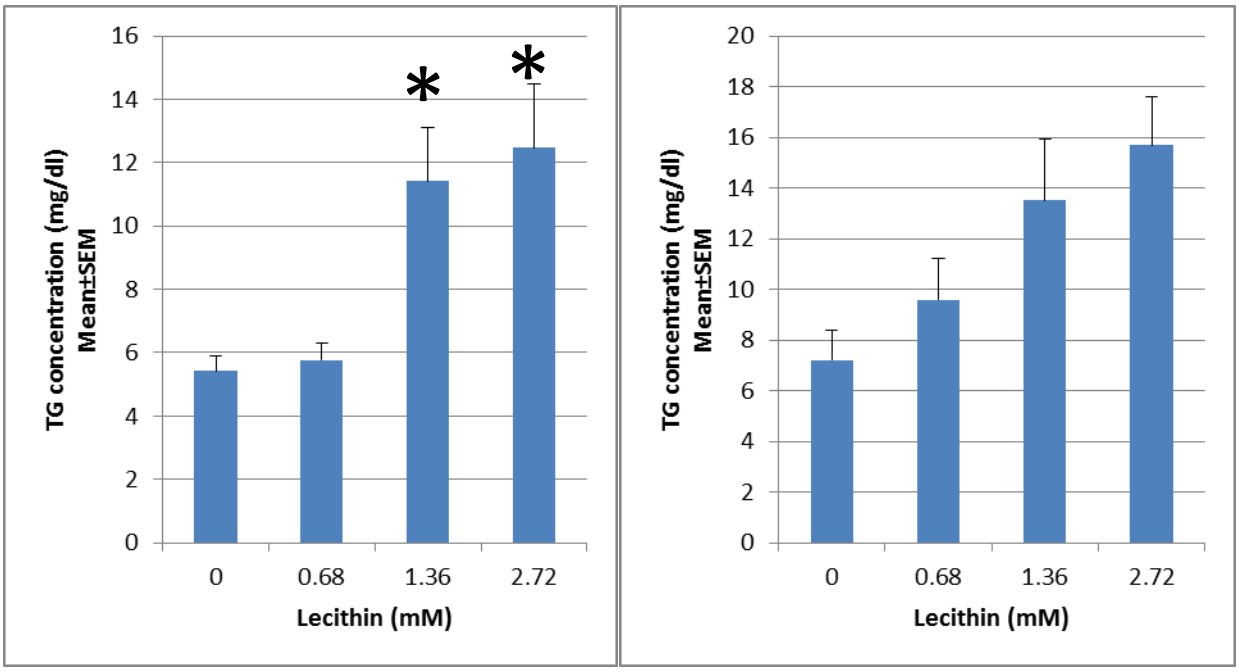


Figure 7. Dose-dependent Effects of Egg Lecithin on Lipoprotein Secretion

Caco-2 cells were grown on 10 cm plates until they reached 13 day postconfluent. The cells were then incubated with varying amounts of lipid. Cells were incubated with 0, 0.68, 1.36, or 2.72 mM egg lecithin in 2 mM OA and 1mM NaTC, After 4 hours of incubation, the cells were washed twice with PBS and replaced with fresh growth media. After 2 hours, the media was collected. The collected media was subjected to sequential NaCl gradient ultracentrifugation and the TG concentrations in their CM (A) ($p= 0.022$) and VLDL (B) ($p= 0.053$) fractions were measured.

TLC Analysis of Egg Lecithin

To detect the presence of lysoPC in lecithin, we performed thin layer chromatography followed by choline staining as shown in Figure 8.

Figure 8. TLC Analysis of Egg Lecithin

Determining the presence of lysoPC in egg-lecithin by TLC. 0.5 mg of lysoPC (left) and 2 mg egg-lecithin (right) in chloroform were separated on silica gel 60 plates using chloroform: methanol: acetic acid: water (50/37.5/3.5/2) (v/v) as the solvent system. The plate was stained by Choline staining (2g potassium iodide, 4 ml acetic acid, 0.34 g bismuth subnitrate in 100 ml total volume) followed by 20% sulfuric acid staining with 225°F heating until the color developed.

Optimizing Chylomicron Secretion: Incubation and Collection Time

As shown in Table 6, timing did significantly affect the VLDL ($p = 0.031$) production but not the CM ($p = 0.068$) production from Caco-2 cells.

Timing Effects on Lipoprotein Secretion		
Incubation + Collection Time (hours)	CM (mg/dl)	VLDL (mg/dl)
2+2	5.37 ± 0.67	6.31 ± 1.71
2+4	9.5 ± 1.91	11.48 ± 0.50
4+2	12.8 ± 1.22	14.53 ± 0.33
4+4	8.68 ± 1.59	12.89 ± 2.33

Table 6: Timing Effects on Lipoprotein Secretion

As shown in Figure 9A and 9B, incubation and collection time did not seem to significantly affect the efficiency of CM ($p = 0.068$), but affected VLDL ($p = 0.031$) secretion significantly. Note that this time frame was chosen because lipoprotein secretion by the enterocytes was more pronounced during this time in both mice (33)and rats (35).

A) B)

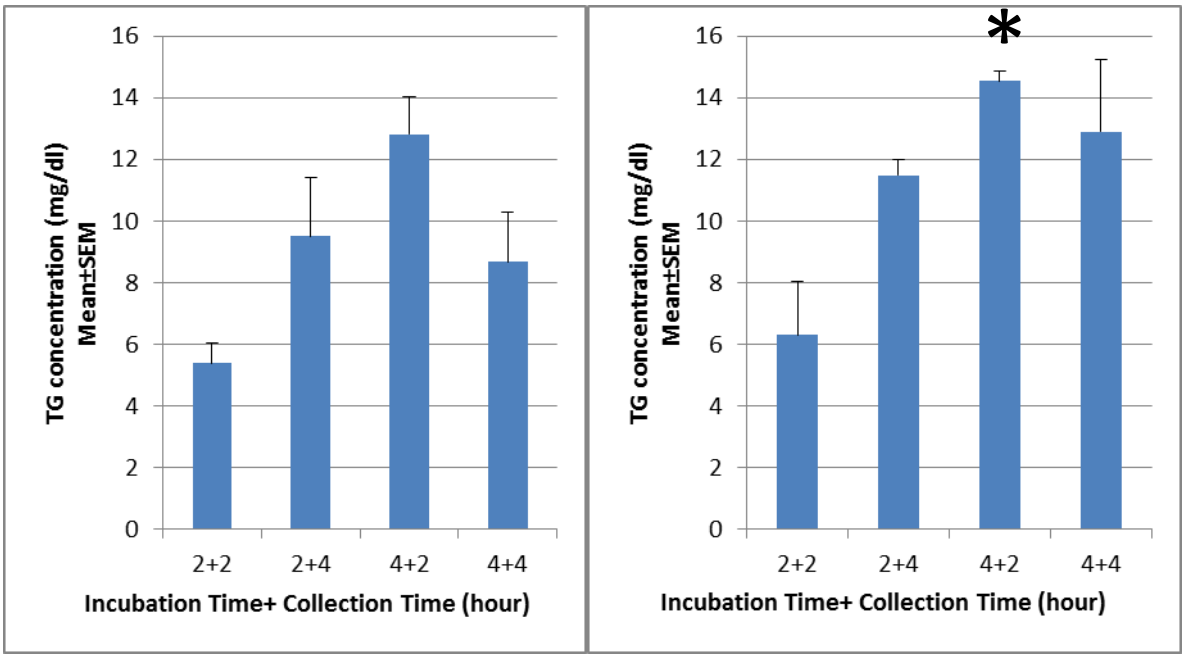


Figure 9. Timing Effects on Lipoprotein Secretion

Caco-2 cells were grown on 10 cm plate until they reached 13 days postconfluent. The cells were then incubated with 4 different incubation and collection combinations were tested. Cells that were at least 13 days postconfluent were incubated with lipid mixture (2mM oleic acid, 1.36 egg lecithin and 1mM NaTC) for relative incubation time. Relative collection time was used to collect lipoproteins. Sequential NaCl gradient ultracentrifugation was conducted to collect chylomicron and VLDL layer. Triglyceride concentrations were measured. Figure 8A was the triglycerides concentration in chylomicron layer ($p=0.068$) and Figure 8B was determined the triglycerides in VLDL layer ($p=0.031$). n =>3

Optimizing Chylomicron Secretion: Collagen Matrix

As shown Table 7, collagen did not significantly affect the production of CM (p= 0.643) or VLDL (p= 0.740) production from Caco-2 cells.

Dose-dependent Effects of Collagen Matrix on Lipoprotein Secretion		
Collagen per plate (µg)	CM (mg/dl)	VLDL (mg/dl)
0	8.933 ± 1.08	17.31 ± 1.08
50	9.47 ± 0.46	18.17 ± 2.65
100	10.56 ± 1.47	16.27 ± 1.48
500	8.58 ± 0.83	15.70 ± 1.16

Table 7: Dose-dependent Effects of Collagen Matrix on Lipoprotein Secretion

As shown in Figure 10, precoating the culture dishes with collagen did not seem to affect the efficiency of CM (p= 0.643) (Figure 10A) or VLDL (p= 0.740) (Figure 10B) secretions. However, the rate of proliferation of Caco-2 cells grown on collagen matrix was noticeably higher relative to the control.

A) B)

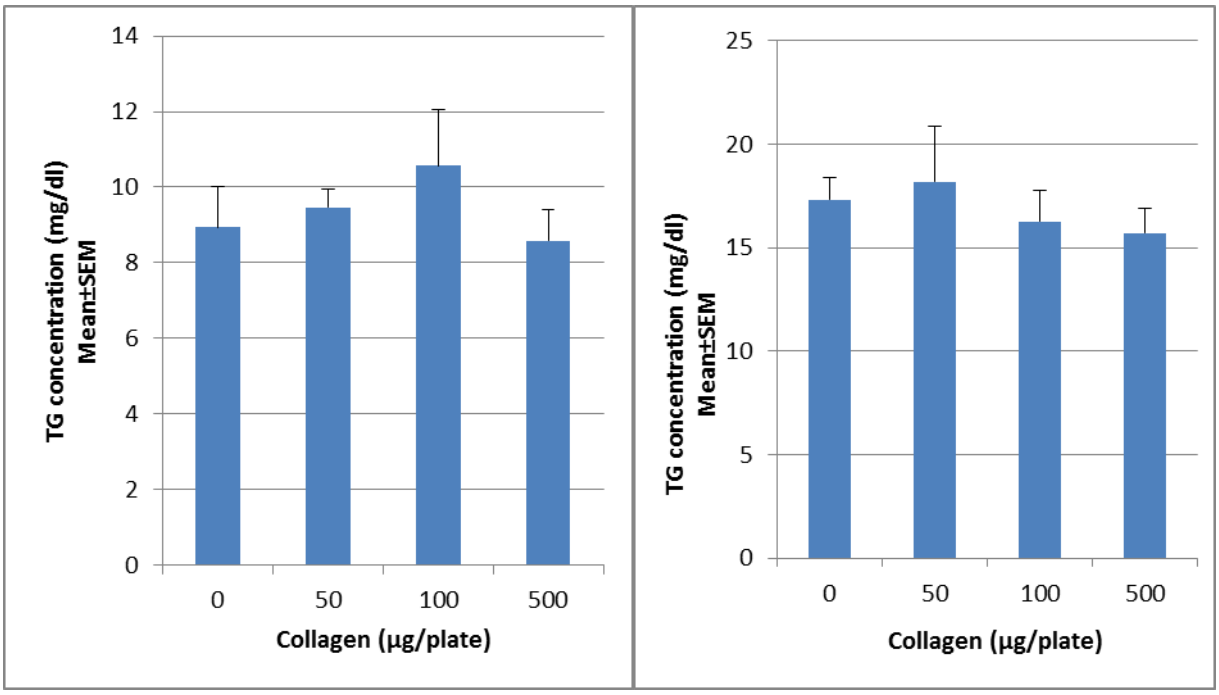


Figure 10. Dose-dependent Effects of Collagen Matrix on Lipoprotein Secretion

Different amounts of collagen (0, 50, 100, 500 µl) were precoated the 10 cm culture dish before cells were growing on. When cells reached 13 days postconfluent, lipid mixture (2mM oleic acid, 1.36mM egg lecithin and 1.0mM NaTC) in 10 ml growth media was added to cells and incubated for 4 hours. After 2 washes with PBS, new growth media was added to collect lipoproteins for 2 hours. Collected media was through sequential NaCl gradient ultracentrifugation to isolate chylomicron and VLDL fractions followed by TG analysis.

Comparison of the Optimal Condition and the Control Groups

As shown in Table 8, both ApoB and TG analysis were used on NL, LL, and O groups. TG concentrations from LL and O groups increased significantly compared with NL group ($p= 0.0019$). ApoB concentrations from LL and O groups also increased significantly compared with NL group ($p= 0.004$).

ApoB and TG Concentrations from CM Fractions of NL, LL, and O Groups		
	ApoB concentration (ng/ml)	TG concentration (mg/dl)
NL	0.62 ± 0.51	0 ± 0
LL	6.77 ± 0.32	6510 ± 784.17
O	8.35 ± 0.09	2833 ± 493.11

Table 8. ApoB and TG Concentrations from CM Fractions of NL, LL, and O Groups

As shown in Figure 11A, CM fractions were collected for NL, LL, and O groups as described in the methods. ApoB measurements (11A) and TG measurements (11B) were tested on each CM fractions from all groups.

A) B)

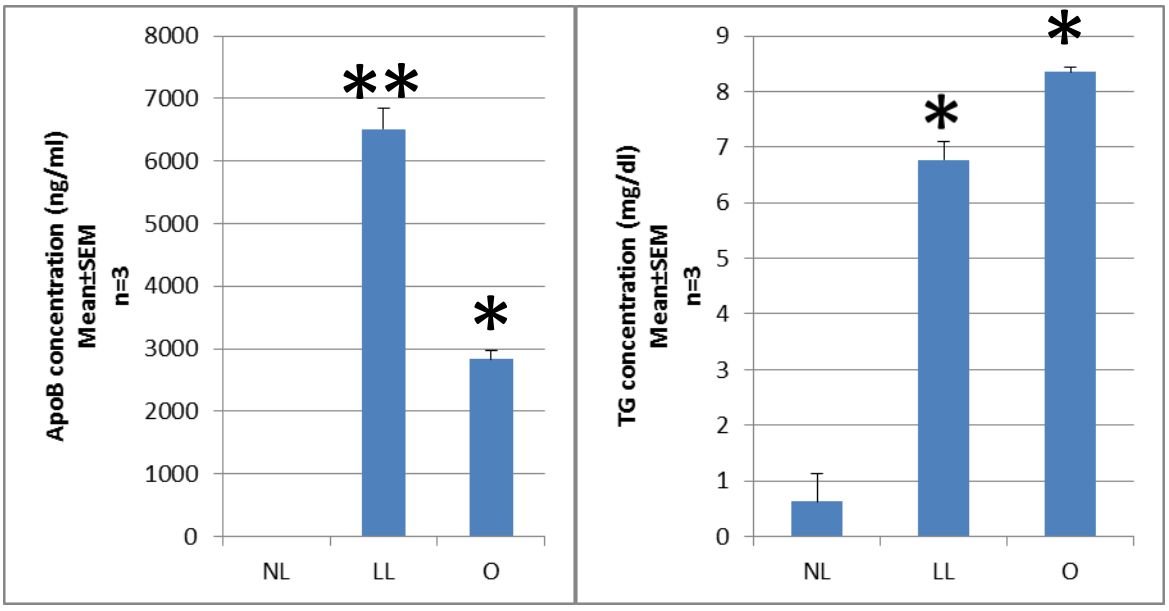


Figure 11. ApoB and TG concentrations from CM fractions of NL, LL, and O Groups

As shown in Table 9, both ApoB and TG analysis were used on VLDL fractions from NL, LL, and O groups. TG concentrations from LL and O groups increased significantly compared with NL group (p= 0.0014). ApoB concentrations from LL group also increased significantly compared with NL and O groups (p= 0.0007).

ApoB and TG Concentrations from VLDL Fractions of NL, LL, and O Groups		
	ApoB concentration (ng/ml)	TG concentration (mg/dl)
NL	1.70 ± 0.17	3666 ± 578.29
LL	10.16 ± 2.16	9590 ± 1161.92
O	9.85 ± 0.77	4282 ± 596.38

Table 9. ApoB and TG Concentrations from VLDL Fractions of NL, LL, and O Groups

As shown in Figure 12, VLDL fractions were collected for NL, LL, and O groups as described in the methods. ApoB measurements (12A) and TG measurements (12B) were tested on each CM fractions from 3 groups. N>= 3

A) B)

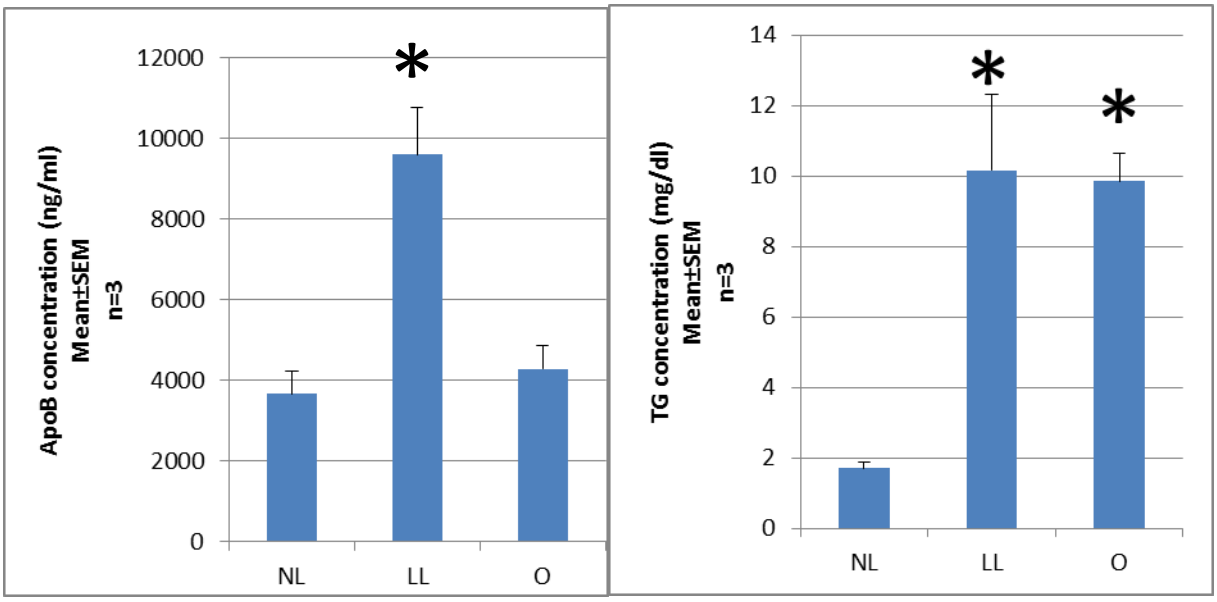
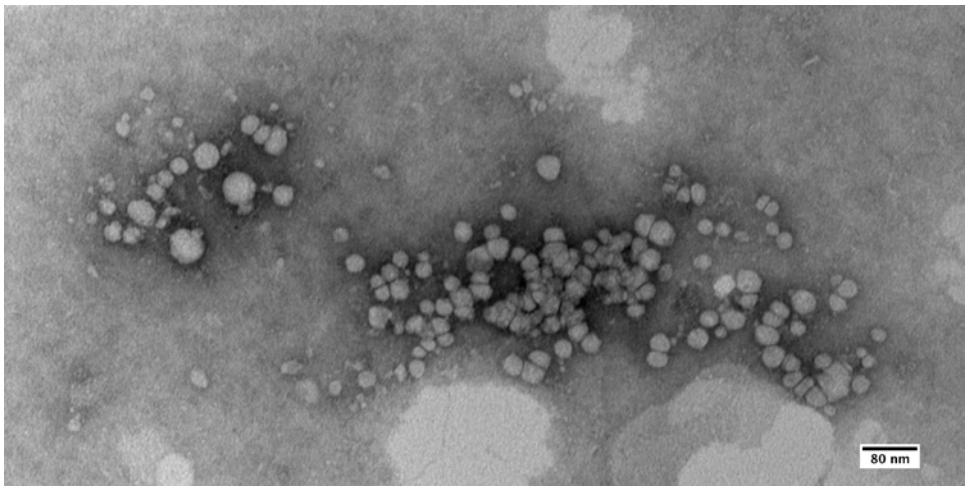


Figure 12. ApoB and TG Concentrations from VLDL Fractions of NL, LL, and O Groups

TEM and Western Blot Analysis on NL, LL, and O Groups

To further confirm the difference between NL, LL, and O groups, TEM and western blot analysis were performed. The lipoprotein of NL (Figure 13A) only consists of VLDLs, while TEM from NL groups (Figure 13B) showed more CM and TEM from O group (Figure 13C) showed more percentage of CM. TEM and histogram based on TEM was used to compare the size distribution and CM percentage. These were also confirmed by histogram (Figure 13D) and CM percent analysis (Figure 13F).

A)



B)

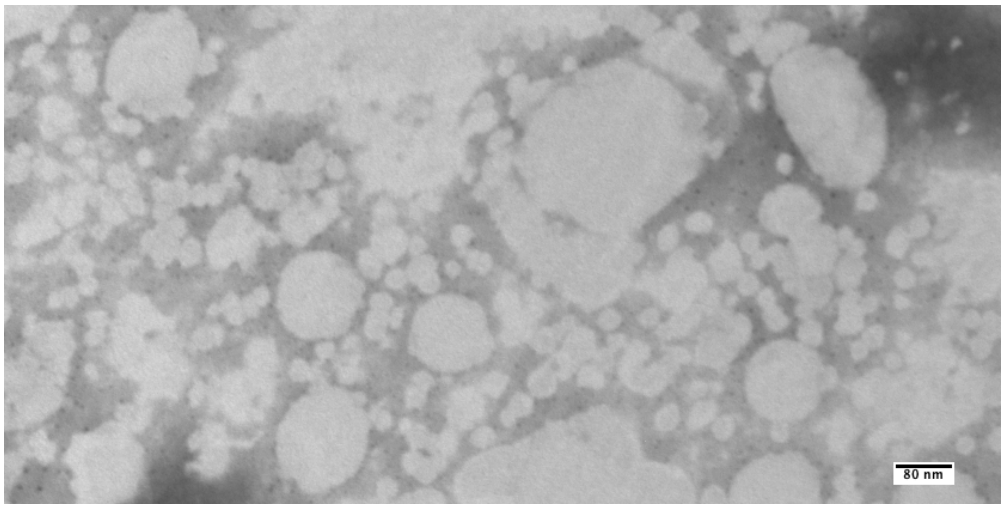
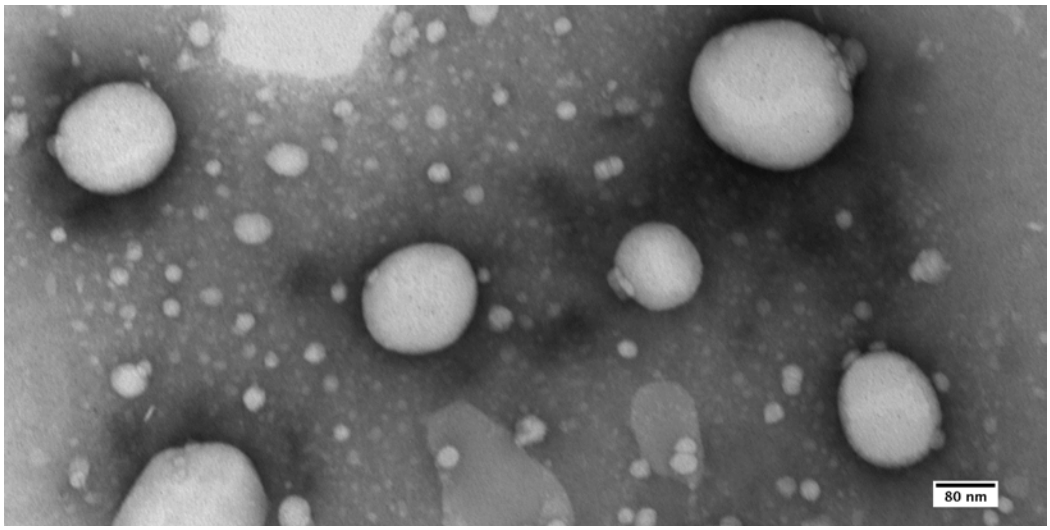


Figure 13 (continued on the next page)

C)



D)

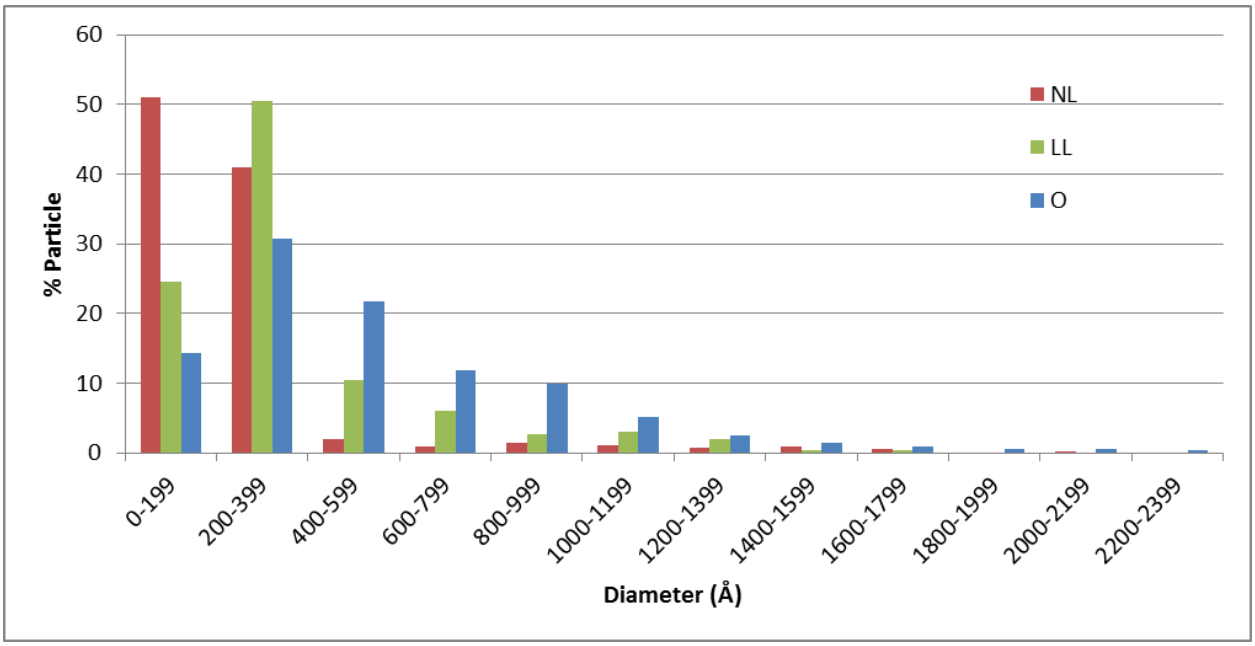
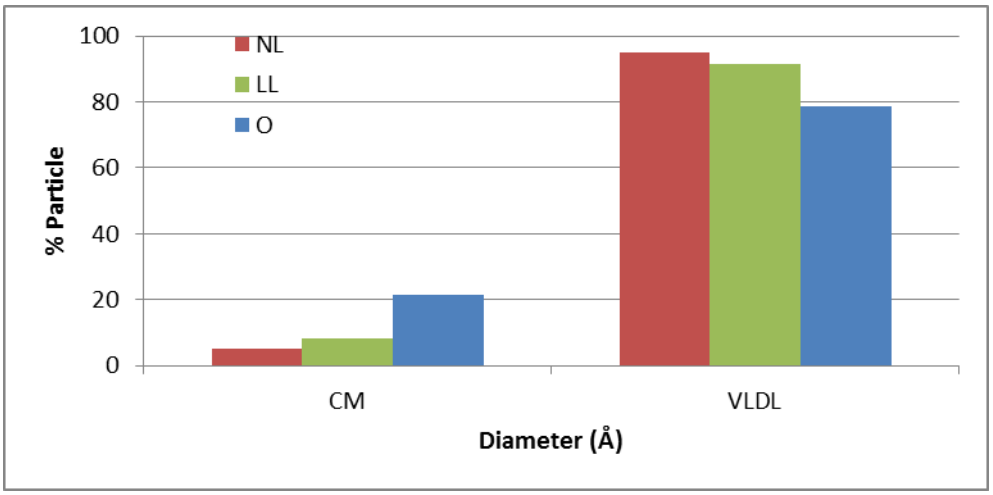


Figure 13 (continued on the next page)

E)



F)

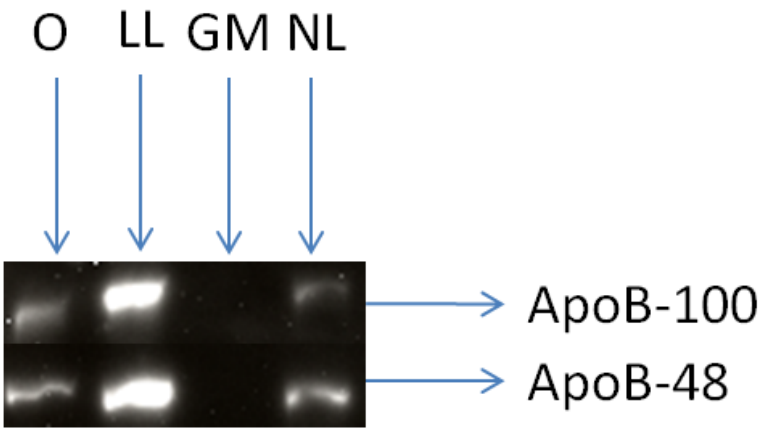


Figure 13. TEM and Western Blot Analysis on NL, LL, and O groups

Caco-2 cells were grown in Transwell until they reached 13 days postconfluence. For NL group, 13 days postconfluent cells were incubated with growth media without lipid in both the apical and basolateral chambers for 4 hours. For LL group, lipid mixture (1.6 mM oleic acids

and 0.5 mM NaTC) in 10 ml growth media was incubated in the apical chamber and growth media without lipid was incubated in the basolateral chamber for 14 hours. For O group, lipid mixture (2 mM oleic acids, 1.36 mM lecithin and 1.0 mM NaTC) in 10 ml growth media was incubated in the apical chamber and growth media without lipid was incubated in the basolateral chamber for 4 hours. Basolateral media collected from NL (Figure 13A), LL (Figure 13B) and O groups (Figure 13C) was subjected to 1-step NaCl gradient ultracentrifugation and analyzed by TEM. In addition, the Apo B in the collected media was also analyzed by Western Blot (Figure 13F). The lipoprotein size distribution study (Figure 13D) and CM percentage (Figure 13E) was based on TEM of 3 groups.

High Performance Lipid Chromatography

One ml fraction of HPLC samples were collected after sample running through the column. Detectable ApoB samples were collected and dialyzed for biochemical analysis like TG assay and ELISA. Although TG and ELISA could detect the lipoproteins from our sample after dialysis, not intact particles were observed in TEM analysis. The particle may not be intact after exposure in room temperature for about a week.

CHAPTER 4

DISCUSSION

Because of the low CM secretion efficiency by Caco-2 cells, sequential gradient ultracentrifugation has been used for CM collection (19, 25, 28). In our study modified sequential NaCl density gradient ultracentrifugation was used in conjunction with Fat Red 7B staining, ApoB and TG measurements and TEM analysis to isolate and analyze CM. Fat Red 7B, known to stain TG, is used to label CM and VLDL to determine their movements during the 2 step NaCl density gradient ultracentrifugation. After the first step of low speed ultracentrifugation, CM was concentrated on the top ~ 1 ml while VLDLs spread evenly below the top fraction. CMs, less dense than VLDL, float to the top faster than VLDL under the same condition. Low speed ultracentrifugation could float CMs to the top layer without floating VLDLs up. The second ultracentrifugation step is to float VLDLs to the top through high speed ultracentrifugation (Figure 1). After the second ultracentrifugation, both top VLDL layer and bottom layer (presumed LDL) were concentrated with heavily stained particles. Through TG and ApoB measurements, we found that CM and VLDL fractions contained similar concentrations of TG while CM fraction contained significantly less lipoproteins. TG and ApoB measurements imply the overall lipoprotein-carrying lipids and the amount of lipoproteins, respectively. The average size of particle, suggested by the average lipids of each particle, can be predicted by the relative amount of TG to ApoB concentrations. Then the fact that less particles in CM fraction carries similar amount of TG suggests the averagely larger lipoproteins in CM fraction than particles in VLDL fraction. This result was further confirmed by TEM analysis (Figure 2). From TEM, we also learned that no CM is found in VLDL fraction which means our sequential NaCl density gradient ultracentrifugation collects all CMs in CM fraction.

Caco-2 cells were examined extensively in lipid studies because differentiated Caco-2 cells displayed enterocyte-like characteristics, including secreting enterocyte-like lipoproteins. Differentiation was critical for Caco-2 cells to display like enterocytes. In early differentiation stage, Caco-2 cells gradually secreted more VLDLs before secreting CMs (Figure 3). Even nondifferentiated Caco-2 cells could secrete VLDLs when transfecting with human ApoB cDNA (27), only well-differentiated cells could secrete CM efficiently. There were 3 stages of differentiation in Caco-2 cells: homogeneously undifferentiated, heterogeneously differentiated, and homogeneously polarized differentiated cells. Studies showed that enterocyte-specific proteins are fully expressed in the heterogeneously differentiated stage in which we also found the optimal ability to secrete CM (Figure 3A). Caco-2 cells proliferated and differentiated upon confluence under standard culture conditions. However, other factors, like collagen and plates material, could affect the growth and differentiation of Caco-2 cells too. Collagen, the main protein fiber of connective tissue, has been shown to accelerate the proliferation of Caco-2 cells (2). However, collagen was not found to have significant effect on either CM or VLDL secretion because the cells for experiment were already well-differentiated (Figure 9). Caco-2 cells were also found to secrete predominantly ApoB-100 if cultured directly on plastic plates. If cultured on semipermeable membrane or filter, Caco-2 cells expressed more ApoB-48 than ApoB-100 (Figure 11F).

Mimicking the conditions of enterocytes, lipid mixture was incubated with cells to induce the production of CM. The lipid hydrolysis product (fatty acid, MG, and lysophospholipid) were reported to induce lipoprotein secretion both from enterocytes and Caco-2 cells. In our study oleic acid was used as the most efficient fatty acid for inducing Caco-2 cells to secrete lipoproteins (8, 11). We found that 2 mM oleic acid has optimal effects on inducing CM

production (Figure 4A). More oleic acid would be toxic to the cells because the hydrophobicity could disrupt the cells membranes, while less oleic acid may not supply sufficient lipids for CMs secretion.

Another lipid hydrolysis product, MG, was reported to promote lipoprotein secretion from enterocytes and could be taken up by Caco-2 cells (16). The dose-dependent effect of mono-olein on CM secretion was tested while no significant effect of mono-olein on lipoprotein secretion was found (Figure 5). The reason could be because Caco-2 cells did not actively use MG pathway for TG assembles but glycerol-3-phosphate pathway (19).

The effects of lysophospholipid effects on CM secretion are also tested by testing egg lecithin for the cost concerns. Through TLC analysis we found the trace of lysophosphatidylcholine (Figure 7), which was reported to promote lipoprotein secretion in Caco-2 cells (12, 31). Significant effects on promoting CM secretion were found. However, the possibility of other components in egg lecithin promoting CM secretion can not be ruled out. Especially phosphatidylcholine, predominant in egg lecithin, is believed to promote lipoprotein secretion from Caco-2 cells also (31).

Two and 4 hours of incubation or collection time were tested because physiological lipoprotein secretion was more pronounced during this time in both mice (33) and rats (35). However, the time variables only made a difference in VLDL secretion (Figure 8). Longer collection time allows cells to secrete more VLDL but not CM. In addition, the longer the incubation and collection time, the more the serum will be consumed.

To determine if our optimal conditions were in fact capable of producing CMs efficiently, we compared it with 2 other groups: less lipid group and no lipid group. Less lipid group used

modified previously studied conditions that were reported to have CM secretion (29)and no lipid group used the same optimal condition except without lipid mixture incubation. Previously studied condition used low lipid in the apical chamber than our optimal condition as well as extreme low serum basolateral media to collect lipoproteins overnight. In less lipid group we changed original extreme low serum basolateral media to normal serum basolateral media. Because when extreme low serum was used in the basolateral media, the TG and ApoB concentrations were too low to be detected in both the CM and VLDL fractions. TEM analysis also confirmed very few of small lipoproteins secreted (data not shown). If Caco-2 cells were incubated with low serum basolateral media for 12 hours like previously reported, cells die and detach from the plates. This suggests that Caco-2 cells need serum in the basolateral chamber to stay healthy and secrete enormous and large lipoproteins. Therefore, we used 15% FBS in DMEM basolateral media for less lipid group instead of 0.1% FBS in DMEM that was used in previous study (28.)

TG, ApoB, and TEM analysis were used to analyze CM and VLDL fractions from 3 groups: Optimal group (our optimal condition), less lipid group (modified previously reported condition), and no lipid group (no lipid mixture added into cells). ApoB concentrations represents the lipoprotein amounts while TG concentration represents overall lipids carried by lipoproteins. Relative amount of TG to ApoB concentration is used to predict the average size of lipoproteins between groups that would be confirmed by TEM analysis.

From CM fraction analysis of 3 groups we found that: no lipid group secretes few CMs suggested by nondetectable ApoB or TG concentrations; Less lipid group secretes the most numerous CM suggested by the highest ApoB concentrations and high TG concentrations; Our optimal group secretes the average largest CM suggested by high ApoB concentrations and high

TG concentrations. All these results are confirmed and supported by our TEM analysis. Similar results were observed in VLDL fraction analysis: no lipid group secretes few VLDLs; less lipid group secretes most numerous VLDLs; optimal group secretes largest VLDLs.

In previous studies ApoB concentrations did not have sufficient amounts for western blot without sensitive methods e.g. radiolabeled lipids (6, 17, 19, 25). Inefficient ultracentrifugation method and poor lipid exportation efficiency probably are the 2 main reasons. With sensitive detective methods, more ApoB-100 was reported to be secreted by Caco-2 cells (24). However, through our gradient ultracentrifugation, ApoB from our optimal condition group, less lipid condition group and no lipid condition group were all detected by direct western blot. And more ApoB-48 was detected than ApoB-100 in all 3 groups. This result supports that more ApoB-48 is secreted when culturing the cells on semipermeable membranes. However, from our condition, higher ratio of ApoB-48 to ApoB-100 was obtained.

In our study we have improved the CM secretion efficiency so that lipoproteins could be detected by biochemical assay and western blot. The presence of CM secretion was analyzed by western blot and confirmed by TEM. And also we improved the percentage of *in vitro* CM secretion efficiency. Overall, 21% of CM secretion efficiency was obtained *in vitro* from our optimal condition, which is close to 39% *in vivo* efficiency (26). One possible reason for low efficiency *in vitro* was that not all VLDLs were collected *in vivo*. In the previous *in vivo* studies, all lipoproteins were assumed to enter lacteal because of the larger gaps between their endothelia cells. However, fenestrated capillaries, thought to have smaller gaps between their endothelia cells, had pores that are about 60-80 nm in diameter. VLDL, which was smaller than the pores, was able to enter the blood capillary. In the animal model lipoproteins were collected from the

lymph. Therefore, some of these VLDLs were not collected, skewing the data toward larger lipoproteins.

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